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Regional Permeability of Salmon Calcitonin in Isolated Rat Gastrointestinal Tracts: Transport Mechanism Using Caco-2 Cell Monolayer

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Rakhi B. Shah,[1] and Mansoor A. Khan[2]

[1]School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter, Amarillo, TX 79106
[2]FDA/CDER/DPQR, White Oak, LS Building 64, HFD-940, 5600 Fishers Lane, Rockville, MD 20895

Abstract

The objective of the study was to determine the region of maximum permeation of salmon calcitonin (sCT) through the gastrointestinal tract and to investigate the mechanism of permeation. For regional permeability determination, male Sprague-Dawley rats (250-300 g) were anesthetized and the gastrointestinal tissues were isolated. Stomach, duodenum, jejunum, ileum, or colon tissues were mounted on Navicyte side-by-side diffusion apparatus. Salmon calcitonin solutions (50 µM in phosphate-buffered saline, pH 7.4, 37°C) were added to the donor side, and the samples were removed from the receiver compartment and analyzed by competitive radioimmunoassay (RIA). For mechanistic studies, Caco-2 cells were grown on Transwell inserts (0.4-µm pore size, 0.33 cm^2 area) in a humidified 37°C incubator (with 5% CO_2). Transport experiments were conducted for sCT solutions (50 µM in Dulbecco's modified eagle's medium [DMEM], pH 7.4) from the apical-to-basolateral (A-to-B) direction and B-to-A direction at 37°C and from the A-to-B direction at 4°C. Cell monolayer integrity was monitored by mannitol permeability and transepithelial electrical resistance (TEER) measurements. The permeability coefficients ($\times 10^{-9}$, cm/sec) for sCT through rat stomach, duodenum, jejunum, ileum, and colon tissues were 0.482 ± 0.086, 0.718 ± 0.025, 0.830 ± 0.053, 1.537 ± 0.32, and 0.934 ± 0.15, respectively. The region of maximum sCT permeability is ileum followed by colon, jejunum, duodenum, and stomach. The permeability coefficients ($\times 10^{-6}$, cm/sec) for sCT through Caco-2 cell monolayer were 8.57 ± 2.34 (A-to-B, 37°C), 8.01 ± 1.22 (A-to-B, 4°C), and 6.15 ± 1.97 (B-to-A, 37°C). The mechanism of its permeation is passive diffusion through the mucosa as determined from the Caco-2 monolayer permeability of sCT.

Keywords: salmon calcitonin, regional permeability, stomach, duodenum, jejunum, ileum, colon, Caco-2.

Corresponding Author: Mansoor A. Khan, FDA/CDER/DPQR, White Oak, LS Building 64, HFD-940, 5600 Fishers Lane, Rockville, MD 20895. Tel: (301) 796-0016. Fax: (301) 796-9816. Email: Khanm@cder.fda.gov.

Introduction

Identifying a region in the intestine that favors peptide and protein absorption is a crucial step in the design of an oral delivery system for these drugs. Regional variation in the penetration barriers to peptides may result in regional differences in their absorption. For instance, M cells located on the dome epithelium of gut-associated lymphoid tissues are known to be capable of sampling macromolecular antigens from the lumen through an endocytic pathway.[1] Insulin and vasopressin have been targeted to large intestine in rats to increase their peroral bioavailability.

Salmon calcitonin is one of the most widely studied peptides for oral delivery and is used for the management of Paget's disease and osteoporosis.[2] Salmon calcitonin needs to be given by daily injections, thus numerous attempts have been made to deliver the peptide orally. For successful oral delivery of sCT, the delivery to its "absorption window" region may lead to a higher bioavailability. In this study, we are interested in identifying the region of maximum permeability in the gastrointestinal tract. For this purpose, we have used rats in our studies.

We are also interested in the transport mechanisms of sCT through human colon adenocarcinoma (Caco-2) cell monolayer. Various enzymes and transporters are known to be expressed in this cell line. Therefore, this cell line has been widely used as an in vitro model for the intestinal mucosa to determine transport characteristics and toxic effects of drugs and to design formulation strategies for membrane permeability enhancement.[3]

Materials and Methods

Materials

Synthetic sCT was purchased from Calbiochem-Novabiochem (La Jolla, CA). Turkey ovomucoid (tOVM) and D-[1-^{14}C] mannitol (specific activity, 43 mCi $mmol^{-1}$; radiochemical purity, 99.3 %) were purchased from Sigma Chemical Co (St Louis, MO). RIA kit for sCT was purchased from DSL laboratories (Webster, TX). Caco-2 cells (C2BBe1 clone), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (PBS), and Trypsin-EDTA were obtained from

American Tissue Culture Collection (ATCC, Rockville, MD). Human transferrin was purchased from Gibco SRL (Los Angeles, CA). All other chemicals were of reagent grade and were used as received.

Transport Studies Through Intestine

Male Sprague-Dawley rats, 250 to 300 g, were used for the permeability experiments. The rats were anesthetized using ketamine-xylazine solution, and the gastrointestinal (GI) tract tissues were isolated by a reported method.[4] The duodenal and ileal segments were removed from top and bottom (13 cm on either side) and the residual small intestine was designated as jejunum. In this study, the central part of the jejunum was used. Colon region was removed following the cecum and was used for the permeability experiment as well.

A side-by-side diffusion apparatus from Trega Biosciences (San Diego, CA) was used. Different tissues were mounted without stripping on a preheated acrylic half-cell, and the cell assembly was then placed in a heated block after joining the other half-cell. The exposed surface area was 1.78 cm^2 and the reservoir volume was 6 mL. The donor and receiver compartments were immediately filled with prewarmed oxygenated PBS. The buffer was circulated by a gas lift (95% O_2-5% CO_2). The flow rate of gas lift was adjusted to 10 ± 2 mL/min^{-1} using a flow meter (ADM 1000, J & W Scientific, Folsom, CA). The tissues were equilibrated for 10 minutes before the drug solution was added to the donor compartment.

Stock solutions of sCT and tOVM were prepared in mucosal buffer. After equilibration of tissues for 10 minutes, drug solution was added on the mucosal side, so that the final concentration of sCT was 50 μM. For the marker studies, ^{14}C-labeled mannitol was added to the donor side giving ~100 000 dpms. The integrity of the tissues was determined by calculating the permeability coefficient (P_{app}) of mannitol.

For jejunum, sCT transport in the presence of inhibitor was also determined. Stock solutions of sCT and tOVM were added on the donor side, so that the final concentration of sCT was 50 μM and that of tOVM was 8.64 μM.

Samples of 500 μL were taken from the serosal side at various times up to 180 minutes and replaced with fresh transport medium. Measurement of mannitol permeability was done by mixing samples (10 μL) with scintillation cocktail (5 mL). ^{14}C dpms were counted using a Beckman LSC6000K liquid scintillation counter. Receiver-compartment sCT was analyzed by competitive RIA method with slight modifications. The standards were diluted in the transport buffer for analysis.

Permeability Studies Through Caco-2 Monolayer

Caco-2 cells were cultured in an atmosphere of 5% CO_2 at 37°C in T-75 tissue culture flasks using DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 3 μL/mL human transferrin. The medium was changed every alternate day until the flasks reached 90% confluence (3-4 days). The cells were seeded at a density of 20 000 cells/well onto polycarbonate Transwell inserts (3-μm pore size, 0.33 cm^2 area) and allowed to grow in a humidified 37°C incubator (with 5% CO_2). Culture medium was changed after every 48 hours.

Transport experiments were conducted on Costar 24-well Transwell plates (Corning, Corning, NY). All transport experiments employed DMEM without any serum or antibiotics as transport medium. Apical chambers contained transport media containing 50 μM of sCT and mannitol, whereas the basolateral chamber contained transport media alone for A-to-B transport. Several experiments were conducted for the drug investigated. These included transport from the A-to-B direction and B-to-A direction at 37°C and from the apical-to-basolateral direction at 4°C.

Prior to the beginning of the experiments, membranes were rinsed with PBS twice and allowed to equilibrate in the transport medium for 2 hours. This step was important to ensure that a constant TEER was obtained, particularly for those plates where experiments were conducted at 4°C. For this purpose, TEER values were frequently monitored. After the equilibration period, the corresponding chamber volume was replaced by drug solutions or controls. At certain time intervals (0, 15, 30, 60, 90, 120, and 180 minutes), samples (50 μL) were taken and replaced with fresh medium.

For all instances, n = 3 for both controls and experimental wells. Samples were appropriately measured and averaged.

Data Analysis

Apparent permeability coefficients (P_{app}, cm/s) of sCT, and ^{14}C-mannitol were calculated using Equation 1.

$$P_{app} = \frac{dM}{dt} \times \frac{1}{AC_0} \times \frac{1}{60} \quad , \qquad (1)$$

where dM/dt is the flux across the tissue (μM min^{-1} or dpm min^{-1}), A is the surface area of the membrane (1.78 cm^2 for Navicyte or 0.33 cm^2 for Transwell insert) and C_0 is the initial drug concentration (50 nmol/mL for sCT or 100 000 dpm/mL for mannitol). The results of experiments performed at least in triplicate are presented as mean ± SD.

Statistical Data Analysis

Statistical data analysis was performed using the Tukey-Kramer Multiple Comparison analysis of variance (ANOVA) with $P < .05$ as the minimal level of significance.

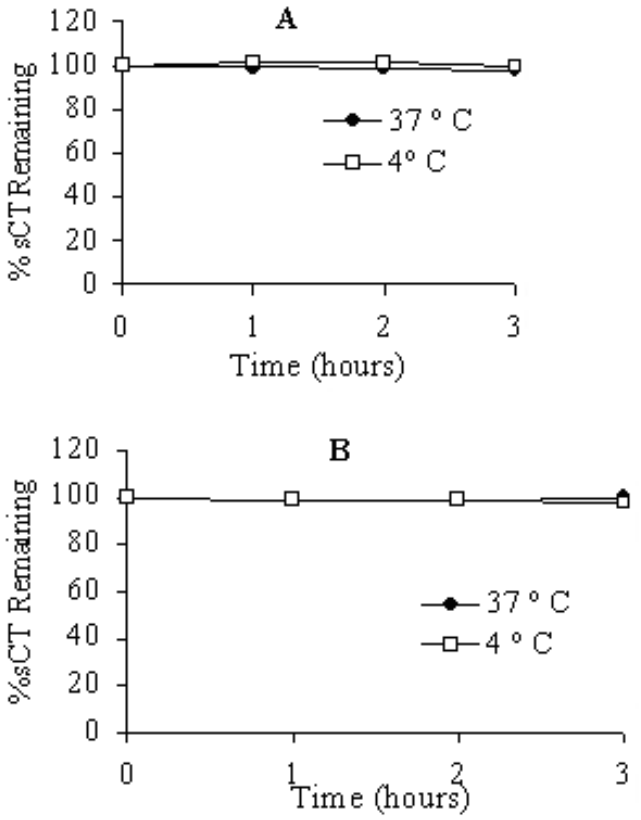

Figure 1. Stability of sCT at 37°C and 4°C in (A) DMEM and (B) PBS.

RESULTS AND DISCUSSION

The absorption site of sCT from 5 different sites of the gastrointestinal tract has been studied. Figure 1 shows the stability of sCT in the transport media for a period of 3 hours. Salmon calcitonin is known to degrade in aqueous media; hence we performed the study to see whether sCT remains stable for the time period for which the study was conducted. It can be revealed from the Figure 1 that sCT remains stable in PBS or DMEM at 37°C and 4°C for a period of 3 hours.

The P_{app} for the different sites in rat gastrointestinal tract are depicted in Figure 2. The P_{app} for sCT were found to be significantly higher than for mannitol as shown in Table 1. The mannitol permeability through these tissues was very low in all cases; hence the tissue remained viable for up to 180 minutes, except in the case of ileum for which the mannitol permeability was found to be higher after 120 minutes. Therefore, the readings after 120 minutes were excluded for the P_{app} calculations in all the cases. P_{app} as shown in Figure

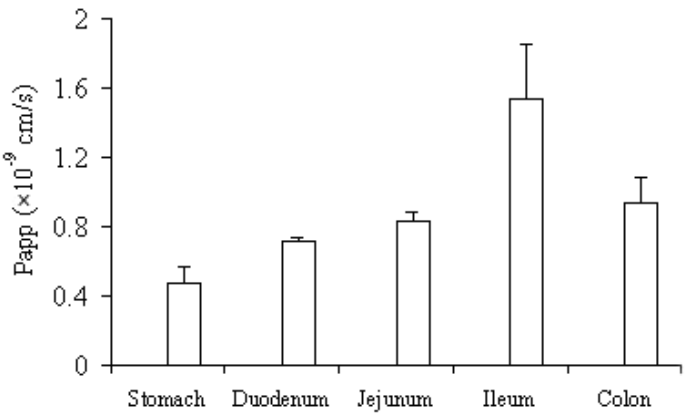

Figure 2. P_{app} $\times 10^{-9}$ cm/s for sCT in rat stomach, duodenum, jejunum, ileum, and colon.

1 and Table 1 are calculated for a period of 0 to 60 minutes. Salmon calcitonin permeation through the rat duodenum was lower than the other small intestinal regions. This finding suggests that there is a regional difference in sCT permeation and hence the absorption. It is striking that the permeation through the colon was lower than the ileum. Low permeability through the colon may be related to the poor membrane permeation as compared with ileum region. The duodenum region has the highest enzymatic activity, and protein/peptide drugs are most poorly absorbed through this region. In this study, we found that sCT permeated least through the duodenum region. This may be because of the enzymatic degradation of sCT in this tissue. The permeation of sCT through the ileum was maximal. It was recently found that the absolute bioavailabilities of sCT following duodenal, ileal, and colonic administration to rats were 0.022%, 0.064%, and 0.021%, respectively.[5] The maximum absolute bioavailability through ileum can be explained on the basis of highest permeability through this region as compared with other regions. There is an evidence of regional differences in bioavailability for human calcitonin (hCT). Human calcitonin was found to have the absolute bioavailability of 0.022% following intraduodenal administration,[5] whereas 0.2% to 0.9% following colonic administration in rats.[6] In human studies, following intracolonic administration, the bioavailability of hCT was from 0.05% to 2.7%.[7,8] Site specificity infers preferential drug absorption at a specific location, usually because of a physiological membrane phenomena or active transport site. A physiological difference in the absorbing surface seems a likely explanation. The factors

Table 1. P_{app} (cm/s) Values for Salmon Calcitonin (sCT) and Mannitol Through Rat Gastrointestinal Tract*

	Stomach	Duodenum	Jejunum	Ileum	Colon
sCT (10^{-9})	0.482 ± 0.086	0.718 ± 0.025	0.830 ± 0.053	1.537 ± 0.32	0.934 ± 0.15
Mannitol (10^{-12})	2.733 ± 0.175	0 ± 0.0112	4.042 ± 2.23	2.645 ± 1.678	135.47 ± 40.80

*The results are expressed as mean ± SD for n = 3. The P_{app} for sCT are given as mean ± SD × 10^{-9} cm/s, whereas the P_{app} for mannitol are given as mean ± SD × 10^{-12} cm/s for various isolated rat tissues.

Table 2. $P_{app} \times 10^{-6}$ (cm/s) Values for sCT and Mannitol Through Caco-2 Cell Monolayer*

	Apical to Basolateral (37°C)	Apical to Basolateral (4°C)	Basolateral to Apical (37°C)
sCT (10^{-6})	8.57 ± 2.34	8.01 ± 1.22	6.15 ± 1.97
Mannitol (10^{-8})	6.551 ± 3.356	4.201 ± 1.987	10 ± 5.978

*The results are expressed as mean ± SD for n = 3. The P_{app} for sCT are given as mean ± SD × 10^{-6} cm/s, whereas the P_{app} for mannitol are given as mean ± SD × 10^{-8} cm/s.

contributing to regional differences in drug absorption include regional differences in the composition and thickness of mucus, pH, surface area, and enzyme activity.[9] The thickness of the mucus layer covering the gastrointestinal tract also shows regional variations. For instance, the mean thickness of the mucus layer in the rat stomach and duodenum are 71 and 81 mm, respectively.[9] This variation might be a reason for higher P_{app} through the duodenum region as compared with stomach. In addition to differences in thickness and composition of mucus and the pH, morphological differences are also seen between different regions of the gastrointestinal tract. The absorptive villi are numerous in the small intestine, becoming fewer and smaller in the more distal sections. This diminishing of villi leads to a progressive decrease in the absorptive surface area per unit serosal length throughout the intestine. For instance, the mucosal area per cm serosal length in the jejunum is ~98 cm^2, while that in the lower ileum is ~20 cm^2.[9] Based on the absorptive surface area in jejunum and ileum, sCT was expected to permeate through the jejunum more than the ileum. However, in the present study, we found that sCT has maximum permeability through the ileum region. The presence of Peyer's patches in ileum might be responsible for the higher permeation of sCT. This indicates that sCT gets absorbed in the lymphatic system and delivery to this region might aid in enhancing its bioavailability through the oral route.

Turkey ovomucoid at a concentration of 8.64 μM was investigated for its effect of sCT permeability through jejunum. Turkey ovomucoid is a protease inhibitor derived from egg white of turkey. It is a glycoprotein with lectin binding as well as inhibitory activity. It was shown to protect sCT against degradation by serine protesases.[10] The concentration was chosen based on its ability to inhibit the serine proteases.[10] The P_{app} for sCT in the presence of tOVM was found to be $1.192 \pm 0.293 \times 10^{-9}$ cm/sec. This was not significantly different from P_{app} of sCT through the rat jejunum in the absence of tOVM (Table 1). This might be because of the removal of intestinal enzymes with washing of the contents of the jejunum. However, it is different from one of the studies in which duck ovomucoid reduced the permeability of insulin through the jejunum.[11] Insulin is believed to be transported through enterocytes by a receptor-mediated process that is blocked by ovomucoids. Transport mechanism of sCT through gastrointestinal mucosal is not known, although it is known to have receptors on bones and gonads.[12,13]

The mechanism of transport of sCT through the epithelial cell monolayer in the gastrointestinal tract was investigated. Transport experiments for sCT through Caco-2 cell monolayer were conducted from the A-to-B direction at 2 temperatures, 37°C and 4°C, and from the B-to-A direction at 37°C. Transport experiments at 2 temperatures provide the information regarding the presence or absence of an active transport mechanism. At low temperatures, active transport mechanisms are decreased significantly. Moreover, transport experiments in 2 directions provide information regarding the presence of an active mechanism as well. The pore size of the Transwell insert did not interfere in the permeation of sCT. This finding was determined by conducting the permeability study of sCT through the insert without the Caco-2 cell monolayer. The transport of sCT in 2 directions and temperatures revealed that there was no significant difference in the permeability coefficient calculated (Table 2). The P_{app} values for sCT in all instances were higher than that of mannitol as shown in Table 2. This finding indicates that sCT is transcellularly permeated through the intestinal membrane, and the differences seen are not because of the paracellular leakage. Mannitol is a highly hydrophilic compound that does not permeate transcellularly. In cases when the tight junctions are disrupted, mannitol shows higher permeability as it leaks through the paracellular route. In this study, we found that the tight junctions were not disrupted for Caco-2 cells. TEER values were monitored for Caco-2 cell monolayer for a period of 3 hours. It was seen in all the cases that TEER remained 100% over that of control, which remained unaffected in all the cases for a period of 3 hours. This finding confirmed that the monolayer retained its integrity for the experimental time span. There are no transporters involved for sCT permeation through the gastrointestinal mucosa; hence a reduction of its permeability was not found in the presence of tOVM. The transport experiments were not conducted further for the elucidation of mechanism as the above directional- and temperature-dependent transport did not give any differences in the P_{app} of sCT. Involvement of active transport mechanism was ruled out based on the P_{app} values obtained for sCT at different temperatures and different directions. If the P_{app} from A-to-B had been found to be higher at 37°C than that of 4°C or that of B-to-A, involvement of active transport would have been involved. In another instance, if the P_{app} from B-to-A had been found to be higher than that of A-to-B, efflux mechanism would have been involved. As no significant differences were seen in the P_{app} values, active transport or efflux mechanisms are not involved in sCT permeation through the GI tract.

The discrepancy between the P_{app} values obtained for isolated rat gastrointestinal tract (Table 1) and Caco-2 cell monolayer (Table 2) could be attributed to the species differences. The Caco-2 cells are epithelial cells derived from human colon. As the rat intestinal tissue and human colonic tissues have different morphological characteristics as well as different thicknesses, it is unlikely to get similar permeability for any compound. Therefore, the absolute permeability values obtained in various animal models should be used with caution as they cannot be directly extrapolated for human.

CONCLUSION

The mechanism of sCT permeation is passive diffusion through the mucosa as determined from the Caco-2 monolayer. There exists a regional difference in the permeability of sCT. The permeation of sCT is maximal through ileum followed by colon, jejunum, duodenum, and stomach. This finding has implications in oral delivery of peptide drugs. Formulation of these compounds into sustained- or controlled-release forms can have a significant impact. If drug release is not complete before the dosage form passes beyond the region of absorption, bioavailability can be severely decreased. Salmon calcitonin can be targeted to the ileum region for increasing the bioavailability through the oral route. The regional permeability of various protein or peptide drugs can be investigated, and the oral dosage form can be tailored to target at the region of their maximum permeation, thereby enhancing oral bioavailability of protein/peptide drugs.

REFERENCES

1. Keljo DJ, Hamilton JR. Quantitative determination of macromolecular transport rate across intestinal Peyer's patches. *Am J Physiol*. 1983;244:G637-G644.

2. Shah RB, Ahsan F, Khan MA. Oral delivery of proteins: Progress and prognostication. *Crit Rev Ther Drug*. 2002;19:135-169.

3. Meunier V, Bourrie M, Berger Y, Fabre G. The human intestinal epithelial cell line Caco-2, pharmacological and pharmacokinetic applications. *Cell Biol Toxicol*. 1995;11:187-194.

4. Asada H, Douen T, Waki M, et al. Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine. *J Pharm Sci*. 1995;84:682-687.

5. Sinko PJ, Smith CL, McWhorter LT, Stern W, Wagner E, Gilligan JP. Utility of pharmacodynamic measures for assessing the oral bioavailability of peptides. 1. Administration of recombinant salmon calcitonin in rats. *J Pharm Sci*. 1995;84:1374-1378.

6. Hastewell J, Lynch S, Williamson I, Fox R, Mackay M. Absorption of human calcitonin across the rat colon in vivo. *Clin Sci*. 1992;82:589-594.

7. Beglinger C, Born W, Muff R, et al. Intracolonic bioavailability of human calcitonin in man. *Eur J Clin Pharmacol*. 1992;43:527-531.

8. Antonin KH, Saano V, Bieck P, et al. Colonic absorption of human calcitonin in man. *Clin Sci*. 1992;83:627-631.

9. Kompella UB, Lee VHL. Delivery systems for penetration enhancement of peptide and protein drugs: Design considerations. *Adv Drug Deliv Rev*. 2001;46:211-245.

10. Shah RB, Khan MA. Protection of salmon calcitonin breakdown with serine proteases by various ovomucoid species for oral drug delivery. *J Pharm Sci*. 2004;93:392-406.

11. Agarwal V, Nazzal S, Reddy IK, Khan MA. Transport studies of insulin across rat jejunum in the presence of chicken and duck ovomucoids. *J Pharm Pharmacol*. 2001;53:1131-1138.

12. Nishikawa T, Ishikawa H, Yamamoto S, Koshihara Y. A novel calcitonin receptor gene in human osteoclasts from normal bone marrow. *FEBS Lett*. 1999;458:409-414.

13. Dong YL, Vegiraju S, Chauhan M, Yallampalli C. Expression of calcitonin gene-related peptide receptor components, calcitonin receptor-like receptor and receptor activity modifying protein 1, in the rat placenta during pregnancy and their cellular localization. *Mol Hum Reprod*. 2003;9:481-490.